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FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 220572US0XPCT

U S APPLICATION NO. (IF KNOWN, SEE 37 CFR): 10/069961

INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/FR00/02494	8 September 2000	10 September 1999

TITLE OF INVENTION

ACELLULAR IMMUNOGENIC COMPOSITIONS AND ACELLULAR VACCINE COMPOSITIONS AGAINST BACILLUS ANTHRACIS

APPLICANT(S) FOR DO/EO/US

MOCK Michele

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☒ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

Request for Priority
Form PTO-1449
Drawings (3 sheets)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/069961	PCT/FR00/02494	220572US0XPCT

24. The following fees are submitted:.	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :		
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**		
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$890.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$130.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	- 20 =	0	x $18.00	**$0.00**	
Independent claims	- 3 =	0	x $84.00	**$0.00**	
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$1,020.00**	
☐ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				**$0.00**	
SUBTOTAL =				**$1,020.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$1,020.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				**$0.00**	
TOTAL FEES ENCLOSED =				**$1,020.00**	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of **$1,020.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **15-0030** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Surinder Sachar
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SIGNATURE

Norman F. Oblon
NAME

24,618
REGISTRATION NUMBER

March 8 2002
DATE

220572US-0X PCT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF: :

MICHELE MOCK : ATTN: APPLICATION DIVISION

SERIAL NO: 10/069,961 :

FILED: March 8, 2002 :

FOR: ACELLULAR IMMUNOGENIC COMPOSITIONS AND ACELLULAR VACCINE COMPOSITIONS AGAINST BACILLUS ANTHRACIS

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Prior to examination on the merits, please amend the above-identified application as follows.

IN THE CLAIMS

Please amend the claims as shown in the marked-up copy following this amendment to read as follows.

4. (Amended) The immunogenic composition as claimed in claim 1, characterized in that it also comprises at least one detoxified exotoxin chosen from the group consisting of the lethal factor (LF) and the edematogenic factor (EF), which have been detoxified.

5. (Amended) The immunogenic composition as claimed in claim 1, characterized in that the spores are derived from a strain of *B. anthracis* chosen from the group consisting of the following strains: Sterne 7702, RPLC2 (Collection Nationale de Cultures et de Microorganismes [National Collection of Cultures and of Microorganisms] held by the Institut Pasteur under the number I-2270, dated July 28, 1999) and RP42 (Collection Nationale de Cultures et de Microorganismes held by the Institut Pasteur under the number I-2271, dated July 28, 1999).

6. (Amended) The immunogenic composition as claimed in claim 1, characterized in that the protective antigen is chosen from the group consisting of the purified protective antigens derived from any wild-type or mutated Sterne strain of *B. anthracis*, and the recombinant protective antigens.

Please add the following new claims.

12. (New) The vaccine composition of claim 3, wherein said vaccine composition further comprises at least one detoxified exotoxin selected from the group consisting of the lethal factor (LF) and the edematogenic factor (EF), which have been detoxified.

13. (New) The vaccine composition of claim 3, wherein the spores are derived from a strain of *B. anthracis* chosen from the group consisting of the following strains: Sterne 7702, RPLC2 (Collection Nationale de Cultures et de Microorganismes [National Collection of Cultures and of Microorganisms] held by the Institut Pasteur under the number I-2270, dated July 28, 1999) and RP42 (Collection Nationale de Cultures et de Microorganismes held by the Institut Pasteur under the number I-2271, dated July 28, 1999).

14. (New) The vaccine composition of claim 3, wherein the protective antigen is chosen from the group consisting of the purified protective antigens derived from any wild-type or mutated Sterne strain of *B. anthracis*, and the recombinant protective antigens.

15. (New) The vaccine composition of claim 14, wherein the protective antigen is derived from the RP42 strain (Collection Nationale de Cultures et de Microorganismes [National Collection of Cultures and of Microorganisms] held by the Institut Pasteur under the number I-2271, dated July 28, 1999).

REMARKS

Claims 1-15 are active in the present application. Claims 4-6 have been amended to remove multiple dependencies. Claims 12-15 are new claims. Support for the new claims is found in the original claims. No new matter is added. An action on the merits and allowance of claims is solicited.

Respectfully submitted,

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Marked-Up Copy
Serial No: 10/069,961
Amendment Filed on:
7-19-2002

IN THE CLAIMS

--4. (Amended) The immunogenic composition as claimed in claim 1 [either of claims 1 and 2, or the vaccine composition as claimed in claim 3], characterized in that it also comprises at least one detoxified exotoxin chosen from the group consisting of the lethal factor (LF) and the edematogenic factor (EF), which have been detoxified.

5. (Amended) The immunogenic composition as claimed in claim 1 [either of claims 1 and 2, or the vaccine composition as claimed in claim 3], characterized in that the spores are derived from a strain of *B. anthracis* chosen from the group consisting of the following strains: Sterne 7702, RPLC2 (Collection Nationale de Cultures et de Microorganismes [National Collection of Cultures and of Microorganisms] held by the Institut Pasteur under the number I-2270, dated July 28, 1999) and RP42 (Collection Nationale de Cultures et de Microorganismes held by the Institut Pasteur under the number I-2271, dated July 28, 1999).

6. (Amended) The immunogenic composition [or vaccine composition] as claimed in claim 1 [any one of claims 1 to 5], characterized in that the protective antigen is chosen from the group consisting of the purified protective antigens derived from any wild-type or mutated Sterne strain of *B. anthracis*, and the recombinant protective antigens.

Claims 12-15 (New).--

ACELLULAR IMMUNOGENIC COMPOSITIONS AND ACELLULAR VACCINE COMPOSITIONS AGAINST *BACILLUS ANTHRACIS*

The present invention relates to acellular immunogenic compositions and also to acellular vaccine compositions against *Bacillus anthracis*, and to the uses thereof in human medicine and in veterinary medicine.

Bacillus anthracis (*B. anthracis*), the agent responsible for anthrax, or charbon, is an aerobic spore-forming Gram-positive bacterium.

This agent induces an infection either by intradermal inoculation or by ingestion or inhalation of the spores (Klein F. *et al.*, (1966), *J. Infect. Dis.*, **116**, 1213-138; Friedlander A.M. *et al.*, (1993), *J. Infect. Dis.* **167**, 1239-1242), the transformation of which into vegetative cells, encapsulated and toxinogenic forms, allows the bacterium to proliferate and to synthesize its virulence factors.

The inventors have recently shown, in a murine model of pulmonary infection with *B. anthracis*, that alveolar macrophages are the primary site of the germination, which is rapidly followed by the expression of the toxin genes, confirming that the encounter between the spore and the host is crucial for the pathogenicity of *B. anthracis* (Guidi-Rontani E; *et al.*, *Molecular Biology*, (1999), **31**, 9-17).

The main virulence factors are:

- the antiphagocytic capsule consisting of poly-γ-*D*-glutamic acid (Avakyan A.A. *et al.* (1965), *J. of Bacteriology*, **90**, 1082-1095) and
- three protein factors which act in paired combination. The edematogenic toxin (PA-EF) induces an edema after subcutaneous injection, whereas the lethal toxin (PA-LF) is responsible

for animal death after intravenous injection (J.W. Ezzell *et al.*, (1984), *Infect. Immun.*, **45**, 761-767). The factor present in both combinations is the protective antigen (PA) which is involved in the binding of toxins to the target cells. The other two factors, the edematogenic factor (EF) and the lethal factor (LF), are responsible for the manifestation of the toxic effect.

The simultaneous production of the capsule and of the of the toxins is essential for the manifestation of the pathogenic power.

The genes encoding the enzymes which synthesize the capsule are carried by the pXO2 plasmid (Green B.D. *et al.*, (1985), *Infect. Immun.*, **49**, 291-297; Uchida I. *et al.*, (1985), *J. Gen. Microbiol.*, **131**, 363-367) and the three genes *pag*, *cya* and *lef*, which encode, respectively, the PA, EF and LF factors, are carried by the pXO1 plasmid, which was described by Mikesell P. *et al.* (*Infect. Immun*, (1983), **39**, 371-376).

Although many studies have shown that PA is the main antigen responsible for protection in the context of natural immunization or immunization acquired by vaccination, the inventors have shown that LF is also a powerful immunogen (Mock M. *Annales de l'Institut Pasteur* [Annals of the Pasteur Institute] December 1990).

In order to clarify the role of the toxin components in the toxicity of *B. anthracis*, the inventors have constructed various mutants. Thus, they have characterized a strain which lacks the pXO2 plasmid and lacks PA by modification of the pXO1 plasmid. Due to the absence of PA, this strain is no longer lethal in nature (Cataldi A. *et al.* (1990), *Molecular Microbiology*, **4**, 1111-1117).

In order to investigate the elements which may be involved in immunization against infection with *B. anthracis*, the inventors have constructed mutants lacking at least one of the toxicity factors responsible for pathogenicity, i.e. deficient in PA, in EF or in LF, or even lacking the pXO1 plasmid and also lacking the pXO2 plasmid. Although lacking toxicity or exhibiting attenuated toxicity, the single mutants, in particular RP9 (EF-) (Collection Nationale de Cultures et de Microorganismes [National Collection of Cultures and of Microorganisms] held by the Institut Pasteur under the number I-1094, dated May 2, 1991) and RP10 (LF-) (Collection Nationale de Cultures et de Microorganismes held by the Institut Pasteur under the number I-1095, dated May 2, 1991), and the double mutant RP 42 (Collection Nationale de Cultures et de Microorganismes held by the Institut Pasteur under the number I-2271, dated July 28, 1999) proved to be capable of producing antibodies immunoprotective against infection with a wild-type Sterne strain. These mutants are described in international application No. 92/19720, and in the articles by C. Pezard *et al.*, (*Infection and Immunity*, (1991), **59**, 3472-3477 and *J. General Microbiology*, (1993), **139**, 2459-2463).

Currently, the veterinary vaccine marketed (Mérial®) is a live vaccine composed of a suspension of spores of the Sterne strain of *B. anthracis*. Its protective efficacy in animals varies depending on the batch, without it being possible to determine the causes of these variations.

This random efficacy, side effects and also the potential risk of disseminating live germs in the environment make its use in humans impossible.

In human medicine, two vaccines against anthrax, essentially developed in Great Britain and in the United States, are used. They are acelullar vaccines

consisting mainly of the protective antigen (PA), prepared from culture supernatants of the toxinogenic Sterne strain of *B. anthracis*, and of an adjuvant which can be used in human medicine, aluminum hydroxide.

Recent studies on these two vaccines have shown that the British vaccine, containing traces of EP and of LF which induce an antibody response by ELISA, is more efficacious in guinea pigs than the American vaccine, which apparently lacks these two components (Turnbull P.C. *et al.*, (1991), *Vaccine*, **9**, 533-539).

However, these two vaccines have a certain number of drawbacks:

- the vaccination protocol is restrictive, since it requires six injections in eighteen months, followed by one booster per year,
- they induce harmful side effects which limit their use,
- the protection induced by these acellular vaccines in animals, against a challenge with a virulent strain, is never complete, unlike that obtained with the live vaccine.

Given the magnitude of the infections caused by *B. anthracis*, many studies are currently dedicated to improving the vaccine so that it does not have the drawbacks set out above, but at the same time exhibits the same protection as the live vaccine.

In this context, the inventors have given themselves the aim of providing a reliable efficacious acellular vaccine free of side effects which overcomes the drawbacks of the existing vaccines and the vaccine properties of which are easy to control.

Consequently, a subject of the present invention is an acellular immunogenic composition capable of inducing an immune response against *B. anthracis* infections,

characterized in that it comprises:

- a protective antigen (PA),
- killed, optionally purified, spores obtained either from mutant strains of *B. anthracis* carrying one or more mutations chosen from mutations in at least one gene encoding a protein responsible for a toxic effect, in *B. anthracis*, or from mutant strains of *B. anthracis* lacking at least one of the pXO1 and pXO2 plasmids,

combined at least with a pharmaceutically acceptable vehicle.

In an advantageous embodiment of the invention, said acellular immunogenic composition is capable of producing antibodies against *B. anthracis*.

A subject of the present invention is also an acellular vaccine composition against *B. anthracis*, characterized in that it comprises:

- a protective antigen (PA),
- killed, optionally purified, spores obtained either from mutant strains of *B. anthracis* carrying one or more mutations chosen from mutations in at least one gene encoding a protein responsible for a toxic effect, in *B. anthracis*, or from mutant strains of *B. anthracis* lacking at least one of the pXO1 and pXO2 plasmids,

combined at least with a pharmaceutically acceptable vehicle and with at least one adjuvant.

For the purpose of the present invention, the term "acellular" means that the immunogenic or vaccine composition no longer contains any viable cells (killed spores).

The adjuvants used are adjuvants conventionally used and will, in particular, be either saponin, in the case of the veterinary vaccine, or advantageously chosen from the group consisting of aluminum hydroxide and

squalene, in the case of the human vaccine.

In the context of the present invention, the spores may be killed by any physical or chemical means which leads to their inactivation. By way of example, mention may be made of treatment with formaldehyde or irradiation.

For the purpose of the present invention, the term "mutation" is intended to mean a deletion, modification or addition in the gene concerned, which results in a gene either lacking its ability to produce the corresponding protein or capable of producing an inactive protein.

According to a particular embodiment of the invention, the immunogenic compositions and the vaccine compositions may also comprise at least one detoxified exotoxin chosen in particular from the group consisting of the lethal factor (LF) and the edematogenic factor (EF), which have been detoxified, i.e. which have lost their toxic properties.

These inactivated protein factors may in particular be obtained by expressing the genes which have been mutated in the sequence encoding the active site of said protein factors (*cya* or *lef*).

The immunogenic and vaccine compositions according to the invention have, surprisingly, a strong protective capacity, of the order of 100%, which is clearly greater than that obtained with the PA alone or the killed spores alone, which makes it possible to obtain complete immunization with a single injection under the conditions for the veterinary vaccine, and two injections under the conditions for the vaccine for human use.

According to another advantageous embodiment of the immunogenic and vaccine compositions according to the

invention, the spores are derived from a strain of *B. anthracis* chosen from the group consisting of the following strains: Sterne 7702 (M. Sterne *J. Vet. Sci. Anima. Indust.*, (1939), **13**, 315-317), RPLC2 (Collection Nationale de Cultures et de Microorganismes held by the Institut Pasteur under the number I-2270, dated July 28, 1999) and RP42 (Collection Nationale de Cultures et de Microorganismes held by the Institut Pasteur under the number I-2271, dated July 28, 1999).

In another advantageous embodiment of the immunogenic and vaccine compositions according to the invention, the protective antigen is chosen from the group consisting of the purified protective antigens derived from any wild-type or mutated Sterne strain of *B. anthracis*, and the recombinant protective antigens, in particular that produced by *B. subtilis*.

Advantageously, the protective antigen is derived from the RP42 strain (Collection Nationale de Cultures et de Microorganismes held by the Institut Pasteur under the number I-2271, dated July 28, 1999).

The subject of the present invention is also the RPLC2 strain deposited with the Collection Nationale de Cultures et de Microorganismes held at the Institut Pasteur under the number I-2270, dated July 28, 1999).

A subject of the present invention is also the use of at least one antibody directed against the spores derived from strains obtained either from mutant strains of *B. anthracis* carrying one or more mutations chosen from mutations in at least one gene encoding a protein responsible for a toxic effect, in *B. anthracis*, or from mutant strains of *B. anthracis* lacking at least one of the pXO1 and pXO2 plasmids, for producing a medicinal product capable of inducing passive immunization. In fact, antibiotics are the only current treatment against anthrax and must be

administered early, before the appearance of the toxic shock. Consequently, a serotherapy aimed at both the toxins and the spore germination would be a good addition.

The antibodies may be polyclonal antibodies obtained by immunizing a suitable animal with the spores derived from strains used for preparing the compositions according to the invention, under conventional conditions for preparing such antibodies.

The antibodies may be monoclonal antibodies obtained in a way known per se, in particular by fusing spleen cells from mice immunized with an antigen consisting of spores derived from strains used for preparing the compositions according to the invention.

A subject of the present invention is also purified antigenic preparations, characterized in that they are derived from *B. anthracis* spores and comprise, for example, one or more of the exoantigens (proteins of spores and of the exosporium) of respective molecular weights 15 kDa, 30 kDa, 55 kDa, and greater than 200 kDa, said molecular weights being determined using the *AMERSHAM® LMW Electrophoresis Calibration Kit*.

In accordance with the invention, the antigenic compositions are obtained by conventional techniques known to those skilled in the art.

The subject of the present invention is also the polyclonal or monoclonal antibodies directed against said antigen compositions.

The immunogenic and vaccine compositions according to the invention may be administered alone or in combination with other vaccines, by injection or by any route conventionally used for vaccination.

The doses to be administered will be determined depending on the animal or the person for whom protection is being sought.

Other characteristics and advantages of the invention appear in the remainder of the description and examples illustrated by the figures in which:

- figure 1 represents the immunoblot analysis of the spore proteins according to the procedure described in example 5,
- figure 2 represents the immunoblot analysis of the exosporium proteins (A) revelation with a polyclonal antibody and a monoclonal antibody (35B8) (B) analysis according to the procedure described in example 5,
- figure 3 represents the various strains of *B. anthracis* used to prepare the RPLC2 strain. The RPLC2 strain produces the toxin components inactivated by point mutations in the active sites of the LF (LF686; H686→a) and EF (EF346/353; K346→Q and K353→Q) protein. In this figure, the numbers which follow Δ indicate the nucleotides at which the deletions begin and end; Erm, Kan and Sps indicate the insertion of erythromycin resistance, kanamycin resistance and spectinomycin resistance cassettes; Ø corresponds to an organism which has no resistance to these antibiotics.

EXAMPLE 1: Materials and methods for preparing the compositions according to the invention

1.1. Construction of the RPLC2 strain

The RPCL2 strain (Collection Nationale de Cultures et de Microorganismes held by the Institut Pasteur under the number I-2270, dated July 28, 1999) is constructed from the strains indicated in figure 3, according to the operating principles described by C. Pezard *et al.* (1993) (reference cited).

1.2 Preparation of PA

The PA protein is prepared from the mutant *B. anthracis* strain RP42 (Collection Nationale de Cultures et de Microorganismes [National Collection of Cultures and of Microorganisms] held by the Institut Pasteur under the number I-2271, dated July 28, 1999).

The medium R culture supernatants (Ristroph J. D. *et al.* (1983) *Infection and Immunity*, **39**, 483-486) are filtered and then concentrated on a Minitan® system (Millipore® PLGC OMP membrane).

The PA antigen is then purified by ultra-rapid chromatography (FPLC) on a monoQ® column according to the protocol described by Pezard C. *et al.* (1993) (reference cited).

1.3. Preparation and inactivation of spores

The spores are prepared from the Sterne 7702 strain according to the procedure described by E. Guidi-Rontani *et al.* (1999) (reference cited).

The spores are prepared on a solid NBY medium and then washed with distilled water. They are inactivated by treatment with formol, at a final concentration of 4%, for 3 hours at 37°C.

After washing by centrifugation, the spores are taken up in the initial volume of physiological saline (final concentration of 10^9 spores/ml).

This suspension is used to perform the immunization.

If necessary, in particular when the intention is to prepare a vaccine for human use, the spores may be purified before the formol treatment, on a 50% to 76% gradient of Radioselectran® (Schering S.A.).

1.4 Preparation of the vaccine compositions

The compositions are prepared either from killed spores alone, prepared according to the procedure described in 1.3, or from a mixture of PA (at a concentration such that 10 µg per mouse are injected) and of killed spores (10^8 spores per mouse), to which either aluminum hydroxide at a final concentration of 0.3% or saponin at a final concentration of 0.05% is added as an adjuvant.

1.5 Protocol for treating mice

Six-week-old female Swiss mice supplied by the company Iffa-Credo (BP0102-69592 L'ARBRESLE-Cedex) are used.

The animals are divided up into groups of six and fed *ad libitum*.

The injections are given subcutaneously into the groin, in a volume of 200 µl.

1.6. Titering antibody levels

The antibody levels are titered using a conventional ELISA assay.

EXAMPLE 2: Effect of two immunizations under the conditions for the human acellular vaccine (protocol No. 1)

2.1. Treatment of animals

The injection protocol for each group is as follows:

- two injections of vaccine compositions prepared as indicated in point 1.4. or of adjuvant (aluminum hydroxide) are given 28 days apart and
- a challenge injection is given on the 43rd day, with the virulent *B. anthracis* strain 17JB (Pasteur reference strain No. 2) provided by the company Rhône-Mérieux.

Four groups of animals are immunized according to this protocol as follows:

- the first group receives the aluminum hydroxide alone (control group),
- the second group receives a PA dose of 10 µg per mouse,
- the third group receives the spores alone, at 10^8 spores per mouse, and
- the fourth group receives the PA + killed spores mixture so as to have 10 µg of PA and 10^8 spores per mouse.

All the groups receive, on the 43rd day, as specified above, a challenge dose corresponding to 30 times the LD50, i.e. 1.5×10^4 spores per mouse.

2.2. Results

The survival rates are given in table I below.

TABLE I

Treatment	Number of deaths at the 43rd day	Percentage survival at the 43rd day
Adjuvant alone	6/6	0%
PA alone	3/6	50%
Killed spores alone	2/6	33%
PA + killed spores	0/6	100%

These results clearly show that only the vaccine compositions according to the invention are capable of allowing complete protection.

EXAMPLE 3: Effect of two immunizations under the conditions for the vaccine for human use (protocol No. 2)

3.1. Treatment of animals

The injection protocol for each group is as follows:

- two injection of vaccine compositions prepared as indicated in point 1.4. or of adjuvant (aluminum hydroxide) are given 21 days apart, and

- a challenge injection is given on the 32nd day, with the virulent *B. anthracis* strain 17JB (Pasteur reference strain No. 2) provided by the company Rhône-Mérieux.

Four groups of animals are immunized according to this protocol as follows:

- the first group receives the aluminum hydroxide alone,
- the second group receives a PA dose of 10 µg per mouse,
- the third group receives the spores alone, at 10^8 spores per mouse, and
- the fourth group receives the PA + killed spores mixture so as to have 10 µg of PA and 10^8 spores per mouse.

All the groups receive, on the 32nd day, as specified above, a challenge dose corresponding to 100 times the LD50, i.e. 1.5×10^4 spores per mouse.

3.2. Results

3.2.1. *Survival rates*

The results are given in table II below

TABLE II

Treatment	Number of deaths at the 32nd day	Percentage survival at the 32nd day
Adjuvant alone	6/6	0%
PA alone	1/6	83%
Killed spores alone	1/7	85%
PA + killed spores	0/6	100%

These results clearly show that only the vaccine compositions according to the invention are capable of allowing complete protection.

3.2.2. *Antibody levels*

The levels of antibodies directed against the spores are high, of the order of 10 000 to 15 000, and identical in the two groups which received them, whether these spores are alone or combined with PA.

These results confirm the synergistic effect of the compositions according to the invention, which, with an antibody level identical to that obtained by injecting the killed spores alone, allows complete protection.

EXAMPLE 4: Comparison of the efficacy of the vaccine compositions according to the invention with the Sterne live vaccine, under the conditions for the vaccine for veterinary use (a single injection using saponin as the adjuvant): challenge with the 17JB strain

4.1. Treatment of animals

The injection protocol for each group is as follows:

- one injection of vaccine composition prepared as indicated in point 1.4. or of saponin is given on D0, and
- a challenge injection is given on the 32nd day, with the virulent *B. anthracis* strain 17JB (Pasteur reference No. 2) provided by the company Rhône-Mérieux.

Five groups of animals are immunized according to this protocol as follows:

- the first group receives saponin alone (control group),
- the second group receives a PA dose of 10 µg per mouse,
- the third group receives the spores alone, at 10^8 spores per mouse,
- the fourth group receives the PA + killed spores mixture so as to have 10 µg of PA and 10^8 spores per mouse, and
- the fifth group receives the Sterne live vaccine

prepared at the Institut Pasteur.

All the groups receive a challenge dose corresponding to 100 times the LD50, i.e. 10^5 spores, on the 32nd day.

4.2. Results

They are given in Table III below.

TABLE III

Treatment	Number of deaths at the 32nd day	Percentage survival at the 32nd day
Adjuvant alone	6/6	0%
PA alone	1/6	83%
Live spores	0/6	100%
Killed spores alone	4/6	33%
PA + killed spores	0/6	100%

These results clearly show that the vaccine compositions according to the invention are as efficacious as the live vaccine and may, consequently, advantageously be used as a veterinary vaccine.

EXAMPLE 5: Immunoblot analysis of the *B. anthracis* spore proteins

5.1. Materials and methods

5.1.1. *Preparation of the polyclonal and monoclonal antibodies*

A polyclonal serum from mice immunized with killed spores derived, for example, from the RPLC2 strain (Collection Nationale de Cultures et de Microorganismes [National Collection of Cultures and of Microorganisms] held by the Institut Pasteur under the number I-2270, dated July 28, 1999) is prepared according to conventional techniques known to those skilled in the art.

The monoclonal antibody specific for the spore surface (35B8) is prepared according to the technique described by Kohler *et al.* (1975), *Nature*, **296**, 495-497.

5.1.2. *Extraction of the spores*

The proteins from the spore are solubilized by treating the spores with a Tris-HCl buffer, at pH 9.8, containing 8M of urea and 2% of SDS, or with a 10 mM Tris buffer at pH 9.5, containing 10 mM of EDTA and 1% of SDS, according to the technique described by Garcia-Patrone (1995), *Molecular and Cellular Biochem.*, **145**, 29-37).

5.2. Results

They are illustrated in figure 1 and figure 2.

The mouse polyclonal serum recognizes 3 protein species of respective molecular weights 15 kDa, 30 kDa, 55 kDa and a protein species of molecular weight greater than 200 kDa (figure 1).

The heaviest species is also recognized by the monoclonal antibody 35B8 and appears to belong to the exosporium (figure 2A).

Specifically, the immunoblot analysis of the exosporium proteins shows that the various monoclonal antibodies used, including 35B8, recognize a protein species of molecular weight greater than 200 kDa (figure 2A).

It emerges from the above that the vaccine compositions according to the invention are capable of allowing complete protection both under the conditions for the human vaccine and under the conditions for the veterinary vaccine.

EXAMPLE 6: Comparison of the efficacy of the vaccine compositions according to the invention administered

according to protocol No. 1 of example 2, with the PA antigen alone, in mice or in guinea pigs: challenge with the 9602 strain

A. Swiss mice

6.1 Treatment of animals:

The injection protocol for each group is as follows:

- two injections of the vaccine compositions prepared as indicated in point 1.4 in example 1 are given 15 days apart (D0 and D15), and
- a challenge injection is given on the 35th day, with the virulent strain 9602 (M. Berthier et al., Lancet, 1996, 347, 9004:828) isolated from a lethal case of human anthrax, and the virulence of which is ten times greater than that of the 17JB strain used in the previous examples; said strain is injected subcutaneously.

4 groups of animals as defined in example 2 are immunized according to this protocol.

All the groups receive, on the 35th day, as specified above, a challenge dose corresponding to 30 times the LD50, i.e. 1.5×10^4 spores per mouse.

6.2. Results

The experiments were repeated 3 times, with different preparations, on batches of 6 to 8 mice per point (due to P3 containment).

The survival rates are illustrated in table IV below.

TABLE IV

Treatment	Percentage survival at the 35th day and up to the 43rd day
Adjuvant alone	0%
PA alone	0%
Killed spores alone	0%
PA + killed spores	100%

B. Guinea pigs

The experiments were carried out twice, on batches of 5 guinea pigs. The protocol is similar to that used in the mice, with the exception of the following points:

- the PA doses are 40 µg per animal,
- the challenge injection is given intramuscularly.

100% survival is obtained for the combination according to the invention, which is killed spores + PA versus 40% in the animals receiving PA alone, which is the composition of the conventional vaccine.

6.3. Antibody levels

These experiments (mice and guinea pigs) were accompanied by monitoring of the antibody response by ELISA on serum samples from mice and from guinea pigs. The anti-PA antibody titers are high (> 5 000); a response of the same order is detected against spore-specific antigens.

EXAMPLE 7: Comparison of the efficacy of the vaccine compositions according to the invention with the Sterne live vaccine, under the conditions for the vaccine for veterinary use as described in example 4 (challenge with the 9602 strain)

The test was carried out on Swiss mice (under the conditions described in example 4). The challenge injection is given with the 9602 strain (M. Berthier et al., mentioned above), to mice which have received a single injection either of live spores (RPLC2) or of

the combination according to the invention, which is killed spores + PA. The protection efficacy, 83%, is identical for both batches.

These results clearly show that it is possible to provide 100% protection of mice and guinea pigs with a vaccine combination comprising killed spores and the PA antigen.

CLAIMS

1. An acellular immunogenic composition capable of inducing an immune response against *B. anthracis* infections, characterized in that it comprises:
   - a protective antigen (PA),
   - killed, optionally purified, spores obtained either from mutant strains of *B. anthracis* carrying one or more mutations chosen from mutations in at least one gene encoding a protein responsible for a toxic effect, in *B. anthracis*, or from mutant strains of *B. anthracis* lacking at least one of the pXO1 and pXO2 plasmids,

   combined at least with a pharmaceutically acceptable vehicle.

2. The acellular immunogenic composition as claimed in claim 1, characterized in that it is capable of producing antibodies against *B. anthracis*.

3. An acellular vaccine composition against *B. anthracis*, characterized in that it comprises:
   - a protective antigen (PA),
   - killed, optionally purified, spores obtained either from mutant strains of *B. anthracis* carrying one or more mutations chosen from mutations in at least one gene encoding a protein responsible for a toxic effect, in *B. anthracis*, or from mutant strains of *B. anthracis* lacking at least one of the pXO1 and pXO2 plasmids,

   combined at least with a pharmaceutically acceptable vehicle and with at least one adjuvant.

4. The immunogenic composition as claimed in either of claims 1 and 2, or the vaccine composition as claimed in claim 3, characterized in that it also

comprises at least one detoxified exotoxin chosen from the group consisting of the lethal factor (LF) and the edematogenic factor (EF), which have been detoxified.

5. The immunogenic composition as claimed in either of claims 1 and 2, or the vaccine composition as claimed in claim 3, characterized in that the spores are derived from a strain of *B. anthracis* chosen from the group consisting of the following strains: Sterne 7702, RPLC2 (Collection Nationale de Cultures et de Microorganismes [National Collection of Cultures and of Microorganisms] held by the Institut Pasteur under the number I-2270, dated July 28, 1999) and RP42 (Collection Nationale de Cultures et de Microorganismes held by the Institut Pasteur under the number I-2271, dated July 28, 1999).

6. The immunogenic composition or vaccine composition as claimed in any one of claims 1 to 5, characterized in that the protective antigen is chosen from the group consisting of the purified protective antigens derived from any wild-type or mutated Sterne strain of *B. anthracis*, and the recombinant protective antigens.

7. The immunogenic composition or vaccine composition as claimed in claim 6, characterized in that the protective antigen is derived from the RP42 strain (Collection Nationale de Cultures et de Microorganismes [National Collection of Cultures and of Microorganisms] held by the Institut Pasteur under the number I-2271, dated July 28, 1999).

8. The RPLC2 strain deposited with the Collection Nationale de Cultures et de Microorganismes

[National Collection of Cultures and of Microorganisms] held at the Institut Pasteur under the number I-2270, dated July 28, 1999.

9. The use of at least one antibody directed against the spores derived from strains obtained either from mutant strains of *B. anthracis* carrying one or more mutations chosen from mutations in at least one gene encoding a protein responsible for a toxic effect, in *B. anthracis*, or from mutant strains of *B. anthracis* lacking at least one of the pXO1 and pXO2 plasmids, for producing a medicinal product capable of inducing passive immunization.

10. A purified antigenic preparation, characterized in that it is derived from *B. anthracis* spores and comprises one or more of the exoantigens of respective molecular weights 15 kDA, 30 kDA, 55 kDA, and greater than 200 kDA.

11. An antibody directed against the antigenic preparations as claimed in claim 10.

Abstract

The invention concerns an acellular immunogenic or vaccine composition for producing antibodies against Bacillus anthracis comprising a protective antigen (PA) and killed and optionally purified spores, obtained from mutating strains of Bacillus anthracis and their uses.

FIGURE 1

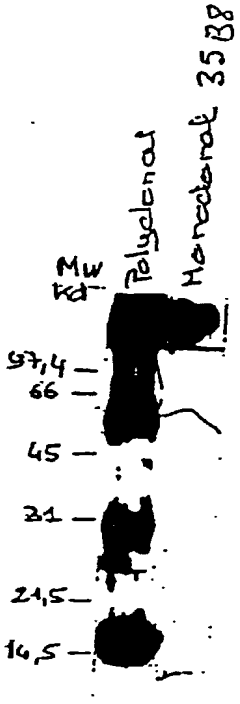

FIGURE 2

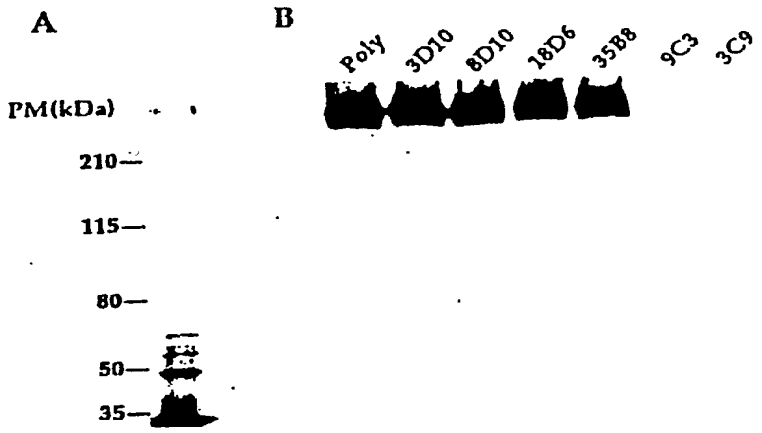

FIGURE 3

Strain	Genotype	Res istance to an antibiotic	Factors produced			Parental strain
7702	pXO1	Ø	PA	LF	EF	Sterne strain (Pasteur collection)
RPA	pXO1-*pagA*(1805-2871)	Spc	—	LF	EF	7702
RPA200	pXO1-*pagA*(1805-4105)	Erm	—	LF	EF	7702
RPL	pXO1-*lefA*(2105-2970)	Spc	PA	—	EF	7702
RPL200	pXO1-*lefA*(405-2911)	Erm	PA	—	EF	7702
RPE	pXO1-*cyaA*(400-2311)	Spc	PA	LF	—	7702
RPE346	pXO1-*cya*346/353	Ø	PA	LF	EF346/353	RPE
RPL686	pXO1-*lef*686	Ø	PA	LF686	EF	RPL
RPL686Δcya	pXO1-*cyaA*(1414-2420)	Kan	PA	LF686		RPL686
RPLC2	pXO1-*lef*686-*cya*346/353	Ø	PA	LF686	EF346/353	RPL686Δcya
RPA163	pXO1-*pagA*(2374-2394)	Ø	PA163	LF	EF	RPA
RPA313	pXO1-*pagA*(2826-2933)	Ø	PA313	LF	EF	RPA
RPA705	pXO1-*pagA*(1005-4053)	Ø	PA705	LF	EF	RPA200
RPA608	pXO1-*pag*608	Ø	PA608	LF	EF	RPA200

Declaration and Power of Attorney for Patent Application
Déclaration et Pouvoirs pour Demande de Brevet
French Language Declaration

En tant l'inventeur nommé ci-après, je déclare par le présent acte que :

Mon domicile, mon adresse postale et ma nationalité sont ceux figurant ci-dessous à côté de mon nom.

Je crois être le premier inventeur original et unique (si un seul nom est mentionné ci-dessous), ou l'un des premiers co-inventeurs originaux (si plusieurs noms sont mentionnés ci-dessous) de l'objet revendiqué, pour lequel une demande de brevet a été déposée concernant l'invention intitulée

et dont la description est fournie ci-joint à moins

☐ ci-joint

☐ a été déposée le

sous le numéro de demande des Etats-Unis ou le numéro de demande international PCT

et modifiée le

(le cas échéant).

Je déclare par le présent acte avoir passé en revue et compris le contenu de la description ci-dessus, revendications comprises, telles que modifiées par toute modification dont il aura été fait références ci-dessus.

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As a below named inventor, I hereby declare that :

My residence, post office address and citizenship are as stated next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed an for which a patent is sought on the invention entitled

Acellular immunogenic compositions and acellular vaccine compositions against *bacillus anthracis*.

the specification of which :

☐ is attached hereto.

☒ was filed on

as United States Application Number or PCT International Application Number.
PCT/FR00/02494 filed on **September 8, 2000**

and was amended on

(if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations,§ 1.56.

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Prior Foreign application(s)
Demande(s) de brevet antérieure(s) dans un autre pays

(Number) (Numéro)	(Country) (Pays)	(Day/Month/Year Filed) (Jour/Mois/Anné de dépôt)	Priority claimed Droit de priorité revendiqué ☒ Yes / Oui	☐ No / Non
99/11384	**FRANCE**	**10/09/1999**		
(Number) (Numéro)	(Country) (Pays)	(Day/Month/Year Filed) (Jour/Mois/Anné de dépôt)	☐ Yes / Oui	☐ No / Non

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(Application No) (N° de demande)	(Filing Date) (Date de dépôt)

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I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1 56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

(Application No.) (N° de demande)	(Filing Date) (Date de dépôt)	(Status) (patented, pending,abandoned) (Statut) (breveté, en cours d'examen, abandonné)
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Je déclare que par le présent acte que toute declaration ci-incluse est, à ma connaissance, véridique et que toute déclaration formulée à partir de renseignements ou de suppositions est tenue pour veridique ;et de plus, que toutes ces déclarations ont eté formulées en sachant que toute fausse déclaration volontaire ou son équivalent est passible d'une amende ou d'une incarcération, ou des deux, en vertu de la section 1001 du Titre 18 du Code de Etats-Unis, et que de telles declarations volontairement fausses risquent de compromettre la validité de la demande de brevet ou du brevet délivré à partir de celle-ci

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French Language Declaration

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POWER OF ATTORNEY : As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to persecute this application and transact all bussiness in the Patent and Trademark Office connected therewith · (list name and registration number)

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(Fournir les mêmes renseignements et la signature de tout co-inventeur supplémentaire.)

(Supply similar information and signature for third and subsequent joint inventors.)